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IS THE PRESENT METHOD OF STANDARDIZING ANTI-DIPHTHERIC SERUM ACCORDING TO ANTI-TOXIN UNITS THERAPEUTICALLY ACCURATE ?*

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CRUVEILHIER¹ quotes Roux, Marfan, Martin, and Momont, as finding that the dose of antidiphtheric serum most efficacious therapeutically is not always the one which contains the greatest number of antitoxic units. The experiences of these authors, Cruveilhier says, seem to indicate that the serum contains, besides the antitoxin, other important preventive and curative substances. In the standardization of antidiphtheric serum as at present practiced, namely, according to antitoxic units, these protective substances are entirely ignored. Cruveilhier to test this point, carried out a series of experiments with guinea-pigs, infected with diphtheria bacilli. He compared sera of different antitoxic strength from several horses as to their value preventively and curatively. In his preventive experiments he injected subcutaneously a quantity of serum proportional to the weight of the animal; and, 24 hours later, inoculated subcutaneously a fatal dose of diphtheria culture. He reported the following results: In four out of seven experiments, with culture No. 261, the animals which received 1/250,000 of 1 c.c., per gram weight, of a 200-unit serum resisted; while those which received the same volume quantity of a 500-unit serum died. In only one instance did the 500-unit serum prove superior, per volume quantity, to the 200-unit serum. Twice the results obtained per volume quantity for both sera were the same. On comparing a 50-unit with one of 500 units, the 50-unit serum proved superior to the 500-unit one, per volume quantity.

With two other cultures, designated "c.c." and "x," he compared a 200-unit serum with a 500-unit one, and obtained practically the

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¹ *Ann. de l'Inst. Past.*, 1904, 18, p. 249.

same results, the 200-unit serum proving more efficacious per volume quantity.

Again, using culture No. 261, he compared a 300-unit serum with one of 500 units, from different bleedings of the same horse, and found them equal per volume in protection.

In his curative experiments, using the same cultures as in the preventive, he injected guinea-pigs subcutaneously with that amount of diphtheria culture which killed the control animal in from 36-48 hours. The animals were divided into two lots. At intervals of two hours, from the second to the 16th hour after inoculation, each animal of one lot received subcutaneously 0.1 c.c. of the serum of the lesser unit content, while each animal of the second lot received 0.1 c.c. of the serum of a greater unit content. In his curative experiments the sera of the lesser unit content were more efficacious than those of the greater.

These results apparently showed that it was the quantity of serum, rather than the number of antitoxic units, which was of therapeutic value.

Cruveilhier drew the following conclusions:

“Que l'effet curatif d'un sérum ne dépend pas exclusivement de sa teneur en unités antitoxiques.

“Que le titrage de l'antitoxine, tel qu'on le pratique habituellement, ne suffit pas à rendre un compte exact de l'efficacité d'un sérum. Que celle-ci est plus exactement appréciée parce que nous avons appelé la mesure du pouvoir thérapeutique.”

In view of the large amount of work which has been done on the titration of diphtheria antitoxin, the results of Cruveilhier cannot but be considered extraordinary.

We have carefully investigated this subject and have obtained results diametrically opposed to those of Cruveilhier and the authors he cites.

In our experiments we compared, both preventively and curatively, native sera and antitoxic globulin solutions (Gibson¹) of the following unit content: A serum of 43 units compared with an antitoxic globulin solution of 1,700 units; a serum of 43 units with an antitoxic globulin solution of 1,450 units; a serum of 200 units with one of 1,000 units;

¹ *Jour. Biol. Chem.*, 1906, 1, p. 161.

a serum of 600 units with one of 1,300 units; a serum of 600 units with one of 335 units, also with one of 200 units. The last three sera were obtained from the same horse during the course of immunization.

The animals infected were active, healthy normal guinea-pigs weighing between 240 and 260 grams.

The diphtheria bacilli were from the three following strains: Culture No. 1, a moderate toxin producer; culture No. 2, a weak toxin producer. Both of these were freshly isolated from the throats of diphtheria patients at the Department of Health Hospital. Culture No. 8 (Park and Williams), is a strong toxin producer, which has been stock culture in this laboratory for 12 years.

In order to avoid misunderstanding, it should be stated that in the first seven preventive, and the first six curative experiments, the cultures in medium-sized tubes were grown on slant agar for 24 hours in the incubator. Of these, $\frac{1}{8}$ of a culture was fatal to 250-gram guinea-pigs in 26 to 33 hours. Beginning with experiment 8 preventive, and with experiment 7 curative, the cultures were grown in *large* uniform test-tubes on slant agar. The fatal dose, thereupon, became $\frac{1}{25}$ culture.

PREVENTIVE EXPERIMENTS.

Following the technique of Cruveilhier, a serum¹ of 43 units per c.c. was compared with an antitoxic solution globulin² of 1,700 units per c.c.

Four guinea-pigs were injected subcutaneously with $\frac{1}{150,000}$, $\frac{1}{200,000}$, $\frac{1}{250,000}$, $\frac{1}{300,000}$ of a c.c. per gram weight, of the 43-unit serum, respectively. A parallel set of four guinea-pigs received the same volume per gram weight of the 1,700 unit antitoxic globulin solution; 24 hours later each animal in both series, as well as a control guinea-pig, received subcutaneously a fatal dose of culture No. 8. The control animal died in $24\frac{1}{2}$ hours. The animals which received the 43-unit serum died in 25–28 hours, while those which received the antitoxic globulin solution remained normal. In the above experiment, the $\frac{1}{150,000}$ c.c. per gram weight of the 43-unit serum contained $\frac{1}{25}$ of a unit; while the same volume of the antitoxic globulin solution contained $2\frac{3}{4}$ units.

¹ Obtained from Horse 308. This animal had received increasing amounts of toxin since March 6, 1906. The most potent antitoxic value was 250 units on May 3, 1906. Although receiving increasing amounts of toxin every eighth day, the potency dropped steadily, and on July 7, 1906, seven days after the last injection of toxin (1,000 c.c. with a M. L. D. of 0.003), the antitoxic value was about 50 units. At the time of this experiment the serum tested 43 units per c.c.

² Prepared from 23 liters of citrated plasma (potency 700 units per c.c.) obtained from four bleedings of Horse 1.

In the second experiment, on account of the great difference in antitoxic unit content, the dilutions were made according to the number of units present.

Four guinea-pigs received $1/6,250$, $1/12,500$, $1/25,000$, and $1/50,000$ c.c. of the 43-unit serum, per gram weight, respectively. Two guinea-pigs received $1/425,000$ and $1/850,000$ c.c. per gram weight, respectively, of the 1,700-unit antitoxic globulin solution. Twenty-four hours later, all the animals, as well as a control, received a fatal dose of culture No. 8. Control animal died in 33–36 hours. The animal which received the $1/50,000$ c.c. of the 43-unit serum showed marked induration and lost $\frac{1}{3}$ of its weight. The two which received the $1/425,000$ and $1/850,000$ c.c. per gram weight of the antitoxic globulin solution remained normal.

In the third experiment made in conjunction with the second, a serum¹ of 200 units was compared with one² of 1,000 units.

Three guinea-pigs received $1/50,000$, $1/100,000$, and $1/200,000$ c.c. of the 200-unit serum, per gram weight, respectively. Three others received $1/250,000$, $1/500,000$, and $1/1,000,000$ c.c. of the 1,000-unit serum, per gram weight, respectively. Twenty-four hours later all the animals, as well as a control, received a fatal dose of culture No. 8. Control animal died in 33–36 hours. The animal which received $1/200,000$ c.c. of the 200-unit serum and the one receiving $1/1,000,000$ of the 1,000-unit serum showed marked induration and great loss of weight.

In the fourth experiment, four guinea-pigs received $1/25,000$, $1/37,500$, $1/50,000$, and $1/75,000$ c.c. of the 43-unit serum, per gram weight, respectively. Four others received $1/1,000,000$, $1/1,500,000$, $1/2,000,000$, and $1/3,000,000$ c.c. of the 1,700-unit antitoxic globulin solution, per gram weight, respectively. Twenty-four hours later all the animals as well as a control received a fatal dose of culture No. 8. Control died in 28 hours. The animal which received the $1/37,500$ c.c. of the 43-unit serum was protected; the $1/50,000$ c.c. failed to

¹ Obtained from Horse 307. This animal had received increasing amounts of toxin since February 14, 1906. The most potent antitoxic value was 350 units on May 3, 1906. Two months later, on July 5, 1906, seven days after the last injection of toxin (850 c.c. with an M. L. D. of 0.003), the antitoxic value was 225 units. At the time of this experiment the serum tested 200 units per c.c.

² Obtained from Horse 305. This animal had received increasing amounts of toxin since February 14, 1906. The most potent antitoxic value was 1,250 units on April 17, 1906. On June 12, 1906, seven days after last injection of toxin (500 c.c. with an M. L. D. of 0.002) the antitoxic value was 1,100 units. At the time of this experiment the serum tested 1,000 units per c.c.

protect. The animal which received the 1/1,500,000 c.c. of the 1,700-unit antitoxic globulin solution was protected; the 1/2,000,000 c.c. failed to protect.

In the fifth experiment, four guinea-pigs received 1/100,000, 1/150,000, 1/200,000, and 1/250,000 c.c. of the 200-unit serum, per gram weight, respectively. Four others received 1/500,000, 1/750,000, 1/1,000,000, and 1/1,250,000 c.c. of the 1,000-unit serum, per gram weight, respectively. Twenty-four hours later all the animals, as well as a control, received a fatal dose of culture No. 8. Control animal died in 28 hours. The animal which received the 1/150,000 c.c. of the 200-unit serum was protected; the 1/200,000 c.c. failed to protect. The animal which received the 1/750,000 c.c. of the 1,000-unit serum was protected; the 1/1,000,000 c.c. failed to protect.

Thus far our results were not in accord with those of Cruveilhier. In our experiments the therapeutic value of the serum appeared to be measured by the antitoxic unit content, rather than, as his experiments indicated, by the quantity of serum.

TABLE 1.
PREVENTIVE EXPERIMENTS 1 TO 5.

EXPERIMENT NO. 1 WITH CULTURE NO. 8				EXPERIMENT NO. 3 WITH CULTURE NO. 8			
Fraction of c.c. per Gram Weight	Serum of Horse 308 43 Units per c.c.	Fraction of c.c. per Gram Weight	Antitoxic Glob. Sol. 1,700 Units per c.c.	Fraction of c.c. per Gram Weight	Serum of Horse 307 200 Units per c.c.	Fraction of c.c. per Gram Weight	Serum of Horse 305 1,000 Units per c.c.
1/150,000	died	1/150,000	lived	1/50,000	lived	1/250,000	lived
1/200,000	died	1/200,000	lived	1/100,000	lived	1/500,000	lived
1/250,000	died	1/250,000	lived	1/200,000	lived	1/1,000,000	lived
1/300,000	died	1/300,000	lived				
Control died in 24½ hrs.				Control died in 33-36 hrs.			
EXPERIMENT NO. 2 WITH CULTURE NO. 8				EXPERIMENT NO. 5 WITH CULTURE NO. 8			
1/6,250	lived	1/425,000	lived	1/100,000	lived	1/500,000	lived
1/12,500	lived	1/850,000	lived	1/150,000	lived	1/750,000	lived
1/25,000	lived			1/200,000	died	1/1,000,000	died
1/50,000	lived			1/250,000	died	1/1,250,000	died
Control died in 33-36 hrs.				Control died in 28 hrs.			
EXPERIMENT NO. 4 WITH CULTURE NO. 8							
1/25,000	lived	1/1,000,000	lived				
1/37,500	lived	1/1,500,000	lived				
1/50,000	died	1/2,000,000	died				
1/75,000	died	1/3,000,000	died				
Control died in 28 hrs.							

In experiment 6, the 43-unit serum was compared with an antitoxic globulin solution¹ of 1,450 units. Four guinea-pigs received $\frac{3}{4}$, $\frac{1}{2}$, $\frac{1}{4}$, and $\frac{1}{8}$ unit of the 43-unit serum, respectively. A parallel set of four guinea-pigs received the same fractions of a unit of the 1,450-unit antitoxic globulin solution. Twenty-four hours later all the animals as well as a control received a fatal dose of culture No. 1. Control animal died in 26 hours. The $\frac{3}{4}$ unit of both the serum and the antitoxic globulin solution protected, while all the animals which received the lower fractions died.

Experiment 7 was identical with the preceding, except that culture No. 2 was used in place of culture No. 1. The results were: Control animal died in 25 hours. The animal which received the $\frac{1}{2}$ unit of the 43-unit serum became greatly emaciated and barely survived. The $\frac{3}{4}$ unit of the same serum failed to protect. In all probability this difference was due to an idiosyncrasy of one of the animals. The $\frac{3}{4}$ unit of the 1,450-unit antitoxic globulin solution protected.

Experiments 6 and 7 were repeated in experiments 8 and 9. The antitoxic values injected were 1, $\frac{3}{4}$, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, and $\frac{1}{16}$ unit. Using culture No. 1 for experiment No. 8, the results were: Control animal died in 26 hours. The $\frac{3}{4}$ unit of both the serum and antitoxin globulin solution protected.

In experiment 9, using culture No. 2, in place of culture No. 1, otherwise parallel with experiment 8, the results were: The control animal died in 23 $\frac{1}{2}$ hours. The one unit of both the serum and the antitoxic globulin solution protected.

In experiment 10, using culture No. 1, a serum² of 600 units was compared with one³ of 1,300 units. The same antitoxic values were administered as in experiments 8 and 9. The results were: Control animal died in 23 hours. The one unit of both sera protected.

In experiment 11, using culture No. 2, in place of culture No.

¹ Prepared from 22 liters of citrated plasma (potency 600 units per c.c.) obtained from two bleedings each of Horses J and L.

² Obtained from Horse N. This animal had received increasing amounts of toxin since October 5, 1906. The most potent antitoxic value was 700 units on February 26, 1907, seven days after the last injection of toxin. Rebleeding one week later, the potency had dropped to 600 units. This was its value also at the time of our experiments, two weeks later.

³ Obtained from Horse 306. This animal had received increasing amounts of toxin since February 14, 1906. The most potent antitoxic value was 1,450 units on May 18, 1906, six days after last injection of toxin (550 c.c. with an M. L. D. of 0.002). At the time of these experiments the serum tested 1,300 units per c.c.

1, otherwise parallel with experiment 10, the results were: Control animal died in 33-36 hours. The $\frac{1}{4}$ unit of both sera barely protected.

In experiment 12, using culture No. 8, in place of No. 2, otherwise parallel with experiments 10 and 11, the results were: Control animal died in 28 hours. The $\frac{3}{4}$ unit of the 600-unit serum protected; the $\frac{1}{2}$ unit of the 1,300-unit serum barely protected.

In the two following experiments (13 and 14) comparisons were made of the three sera¹ of different unit content obtained from the same horse during the course of immunization.

In experiment 13, using culture No. 1, five guinea-pigs received 1, $\frac{3}{4}$, $\frac{1}{2}$, $\frac{1}{4}$, and $\frac{1}{8}$ unit of a 600-unit serum respectively, five others received the same antitoxic unit values of a 335-unit serum; five more received the same values of a 200-unit serum. Twenty-four hours later all the animals, as well as a control, received a fatal dose of culture No. 1. The results were: Control animal died in 28 hours. The $\frac{1}{2}$ unit of each of the three sera protected.

In experiment 14, using culture No. 8 in place of culture No. 1, otherwise parallel with experiment No. 13, the results were: Control animal died in 30 hours. The $\frac{1}{2}$ unit of each of the three sera protected.

To summarize: In experiments 1, 2, 4, 6, 7, 8, and 9, we compared a 43-unit native serum with a 1,700-unit and a 1,450-unit antitoxin globulin solution. The results, in each case, are seen to depend on the antitoxic unit content of the serum and not on its volume. The same is true of experiments 3 and 5, in which a 200-unit serum was compared with one of 1,000 units, and of experiments 10, 11, and 12, in which a fresh serum (two weeks old) of 600 units was compared with one (10 months old) of 1,300 units. Finally the same results are seen in experiments 13 and 14, in which we compared three bleedings from the same horse as follows: One bleeding originally contained 625 units per c.c. (this was the maximum value attained by the animal). The second contained 335 units per c.c., and the third contained 200 units per c.c.

¹ Obtained from Horse 322. This animal had received increasing amounts of toxin since March 7, 1907. The most potent antitoxic value was 625 units, on April 30, 1907. Tested at the time of these experiments the serum contained 600 units per c.c. After the fourth bleeding, which was on July 7, 1907, the antitoxic value had dropped to 340 units; tested at the time of these experiments the serum contained 335 units per c.c. After three further bleedings, the antitoxic value dropped to 200 units on June 28, 1907. This was the potency also at the time of these experiments.

TABLE 2.
PREVENTIVE EXPERIMENTS 6 TO 14.

AMOUNT INJECTED IN UNITS	EXPERIMENT No. 6 WITH CULTURE No. 1		EXPERIMENT No. 10 WITH CULTURE No. 1		EXPERIMENT No. 13 WITH CULTURE No. 1		
	Serum of Horse 308 43 Units per c.c.	Antitoxic Glob. Sol. 1,450 Units per c.c.	Serum of Horse N 600 Units per c.c.	Serum of Horse 306 1,300 Units per c.c.	Serum of Horse 322 600 Units per c.c.	Serum of Horse 322 335 Units per c.c.	Serum of Horse 322 200 Units per c.c.
1 unit			lived	lived	lived	lived	lived
2 " "	lived	lived	died	died	lived	lived	lived
3 " "	died	died	died	died	lived	lived	lived
4 " "	died	died	died	died	died	died	died
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antitoxic globulin solution. The results of both lots were uniform. Control animal died in 25 hours. The animals which received the curative dose after 2 and 4 hours lived; while those receiving the curative dose after 6, 8, and 10 hours, died.

Experiment 2.—The animals were infected with culture No. 1, and divided into two lots. At intervals of two hours, from the second to the eighth hour, each animal of the first lot received subcutaneously 25 units of the 43-unit serum; and each animal of the second lot, 25 units of the 1,450-unit antitoxic globulin solution. The results of both lots were uniform. Control animal died in 26 hours. The animals which received the curative dose after 2, 4, and 6 hours lived; while those receiving the curative dose after 8 hours, died.

Experiment 3.—The animals were infected with culture No. 2, in place of culture No. 1; otherwise parallel with experiment 2. The results of both lots were again uniform. The control animal died in 26 hours. The animals which received the curative dose after 2 and 4 hours lived; after 6 and 8 hours, died.

Experiment 4.—Repeating experiment 2, in duplicate, the result of both lots were again uniform. The control animal died in 21½ hours. The animals which received the curative dose after 2 and 4 hours lived; after 6 and 8 hours, died.

Experiment 5.—Repeating experiment 3, in duplicate, the results were again uniform. The control animal died in 28 hours. The animals which received the curative dose after 2, 4, and 6 hours, lived; after 8 hours, died.

Experiment 6.—The animals were injected with culture No. 1 and divided in two lots. At intervals of two hours, from the fourth to the eighth hour, each animal of the first lot received subcutaneously 25 units of the 600-unit serum. The second lot received 25 units of the 1,300-unit serum. The results of both lots were uniform. The control animal died in 27 hours. The animals which received the curative dose after 4 and 6 hours, lived; after 8 hours, died.

Experiment 7.—The animals were infected with culture No. 2 in place of culture No. 1, otherwise parallel with experiment No. 6. The results of both lots were uniform. The control animal died in 28 hours. The animals which received the curative dose after 4 and 6 hours lived; after 8 hours, died.

TABLE 3.
CURATIVE EXPERIMENTS I TO 11.

HOURS ELAPSED AFTER INJECTION OF CULTURE	EXPERIMENT 1 WITH CULTURE 8		EXPERIMENT 2 WITH CULTURE 1		EXPERIMENT 6 WITH CULTURE 1		EXPERIMENT 9 WITH CULTURE 1		
	Serum of Horse 308 43 Units per c.c. (25 Units Injected)	Antitoxic Glob. Sol. 1,700 Units per c.c. (25 Units Injected)	Serum of Horse 308 43 Units per c.c. (25 Units Injected)	Antitoxic Glob. Sol. 1,450 Units per c.c. (25 Units Injected)	Serum of Horse N 600 Units per c.c. (25 Units Injected)	Serum of Horse 306 1,300 Units per c.c. (25 Units Injected)	Serum of Horse 322 600 Units per c.c. (25 Units Injected)	Serum of Horse 322 335 Units per c.c. (25 Units Injected)	Serum of Horse 322 200 Units per c.c. (25 Units Injected)
2 hours	lived	lived	lived	lived	lived	lived	lived	lived	lived
4 "	died	died	lived	lived	lived	lived	died	died	died
6 "	died	died	died	died	died	died	died	died	died
8 "	died	died	died	died	died	died	died	died	died
10 "	died	died	died	died	died	died	died	died	died
	Control died in 25 hrs.		Control died in 26 hrs.		Control died in 27 hrs.		Control died in 22 hrs.		

Experiment 8.—The animals were infected with culture No. 8, in place of culture No. 2, otherwise parallel with experiments 6 and 7. The results of both lots were uniform. The control died in 27 hours. The animals which received the curative dose after 4 and 6 hours lived; after 8 hours, died.

Experiment 9.—The animals were infected with culture No. 1 and divided into three lots. At intervals of two hours, from the fourth to the eighth hour, each animal of the first lot received subcutaneously 25 units of the 600-unit serum, the second lot, 25 units of the 335-unit serum, and the third lot received the same number of units of the 200-unit serum. These three sera were obtained from the same horse during the course of immunization. The results of the three lots were uniform. The control died in 22 hours. The animals which received the curative dose after 4 hours lived; after 6 and 8 hours, died.

Experiment 10.—The animals were infected with culture No. 8 in place of culture No. 1, otherwise parallel with experiment 9. The results of the three lots were uniform. The control animal died in $24\frac{1}{2}$ hours. Those which received the curative dose after 4 hours lived; after 6 and 8 hours, died.

Experiment 11.—The animals were infected with culture No. 2, in place of culture No. 1; otherwise parallel with experiments 9 and 10. The results of the three lots were uniform. The control died in 27 hours. Those which received the curative dose after 4 and 6 hours lived; after 8 hours, died.

CONCLUSIONS.

In view of the results obtained in the comparison of the antitoxic content of sera, both preventively and curatively, it is obvious that in diphtheria in the guinea-pig, the therapeutic value of antidiphtheric sera depends on the number of antitoxic units present. There is no reason for believing that conditions in man are different.

According to our experiments antidiphtheric serum contains no protective substances, aside from the antitoxin, which play an important rôle therapeutically.

The present method of standardizing antidiphtheric serum accurately measures its therapeutic value.